PHENOTYPIC CHARACTERIZATION OF RHIZOBIA THAT NODULATE BALL CLOVER

A Thesis

by

MARTHA LUCIA CEPEDA HERNANDEZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Soil Science

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Approved by:

Co-Chairs of Committee,	Richard W. Weaver
	Gerald W. Evers
Committee Member,	Alejandro Castillo
Head of Department,	Wayne Smith

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ABSTRACT

Phenotypic Characterization of Rhizobia

That Nodulate Ball Clover. (August 2005)

Martha Lucia Cepeda Hernandez, B.S., Universidad Nacional de Colombia

Co-Chairs of Advisory Committee: Dr. Richard W. Weaver
Dr. Gerald W. Evers

A total of 43 *Rhizobium leguminosarium* bv. *trifolii* isolates were obtained from soil samples of two ball clover (*Trifolium nigrescens*) pastures from Iola and Kilgore (Texas) using ball clover as capture plants. The isolates were phenotypically characterized by intrinsic antibiotic resistance (IAR) against a range of concentrations of eight antibiotics, and by the utilization of 95 different carbon sources (BIOLOG system). The rhizobial isolates were also evaluated for their tolerance to salinity, high temperatures and low pH. The isolates showed two different ranges of growth rates: fast-growing (doubling times between 1.4 - 3.7 h) and slow- growing isolates (12.3 - 21.3 h). The numerical analysis of the phenotypic characteristics showed that the 43 isolates could be grouped in 24 different strains. Cluster analysis based on sensitivity responses of IAR, metabolic profiles of BIOLOG and salt, temperature and acidity tolerance levels could distinguish the *Rhizobium* strains from a *Pseudomonas* isolate. The analysis also showed that the rhizobial strains isolated from ball clover nodules are different from a commercial *R. leguminosarium* bv. *trifolii* strain used as inoculant for this legume.

I dedicate my achievements to my parents, Ricardo and Marina,

because their love and support have been the most enduring forces in my life

And I dedicate my work to the love of my life, Juan,

because he makes all my dreams come true.

This work is also for my abuelita Mercedes,

because you are always looking down at me from Heaven.

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Finally I would like to thank the faculty and staff of the Department of Soil and Crop Sciences, for their continuing cooperation in this research and for their professional treatment of me as more of a friend than a student.

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INTRODUCTION

Soil bacteria of the *Rhizobiaceae* family are able to fix atmospheric nitrogen in symbiosis with important leguminous species such as clovers, increasing their biomass production and adding nitrogen to agricultural systems. Hence, the symbiotic bacteria are of significant agricultural and ecological value especially for agro ecosystems where the quality and amount of forage is a crucial factor in cattle feeding.

Several annual clover species are used throughout the southeastern United States because perennial legumes do not survive the hot dry summers and clovers are more soil specific than grasses. (Evers and Smith, 1998). They extend the grazing season by about 6 weeks, have higher nutritive value than grasses, add nitrogen, and provide some spring weed control (Evers, 2000). Ball Clover (Trifolium nigrescens Viv.) is adapted to fine sandy loam to clay loam soils and can tolerate some poor drainage. Livestock producers like ball clover because it produces seed under grazing with a high percentage of hard seed that enhances volunteer reseeding each autumn. Therefore annual cost of seeding clovers is avoided.

The main limitation to expanded use of ball clover is the difficulty in obtaining stands. Ball clover seed is very small (2.2 million seed kg^{-1}) resulting in weak seedlings. At the present time, rhizobial strains used for white clover (Trifolium repens L.) are recommended for ball clover that results in less than satisfactory nodulation and nitrogen fixation. Therefore, a key role in this research was to obtain rhizobial isolates from ball clover in the agricultural environment in order to provide germplasm for appropriate inoculants.

This thesis follows the style of *Applied and Environmental Microbiology.*

Inoculation with non-native commercial strains of clover rhizobia has been used to improve the production of this crop in several experimental trials (Smith and Gilbert, 1986; Smith et al., 1988; Smith et al., 1989; Smith et al., 1992). However, the yield is not only limited by the N_2 fixing potential of the clover-rhizobia symbiosis but also by the edapho-climatic conditions that have an effect on the adaptation and establishment of the symbiosis (Sessitsch et al., 2002; Howieson and Ballard, 2004). Therefore, soils where the ball clover crop has been effectively established may contain strains well adapted to the environmental conditions that could be isolated and evaluated for use as ball clover inoculants in other soils of East Texas.

This research determines the phenotypic diversity of the rhizobia that nodulates ball clover through their growth characteristics, intrinsic antibiotic resistance and carbon source utilization. This could lead to an improvement in the selection of strains that efficiently nodulate and fix nitrogen and may be used as inoculants for ball clover.

LITERATURE REVIEW

Importance of the genus *Rhizobium*

Plant growth is often limited by the amount of available nitrogen when other soil nutrient deficiencies have been corrected by amendments or fertilizations. The rhizobia are able to supply available nitrogen to the soil by fixing the atmospheric nitrogen gas into organic compounds. The members of the genus *Rhizobium* are non-spore forming Gram negative rods, usually containing poly-β-hydroybutyrate granules observable under phase contrast microscopy (Jordan, 1984). These organisms occur as free-living microorganisms in soil or as microsymbionts in root nodules of leguminous plants (Jordan, 1984).

Rhizobia in root nodules are estimated to carry out between 50 to 70% of the world's biological nitrogen fixation, and the estimated annual biological fixation of atmospheric nitrogen varies between 100 x 10^6 and 180 x 10^6 metric tons per year (Phillips, 1980; Burris and Roberts, 1993). .

Biological nitrogen fixation has of particular importance in agriculture. Leguminous plants that fix nitrogen well may grow on soils that are poor in available nitrogen, reducing the amendments with expensive nitrogen fertilizers (Burris and Roberts, 1993).

Leguminous plants are also of crucial importance as animal feed. Alfalfa and clovers are grown over extensive areas as forage crops for grazing or as dry hay, and they furnish not only high quality protein but also a variety of biologically active molecules such as vitamins, minerals and other nutrients (Burris and Roberts, 1993).

Despite the fact that this process is free, self-sustaining and non-polluting, it does not necessarily operate with optimum efficiency. Nitrogen fixation requires a significant amount of energy by the cell and by the whole plant (Phillips, 1980). It is possible to enhance the nitrogen fixation in the *Rhizobium*-legume symbiosis by selecting host plant phenotypes as well as effective rhizobial strains.

Although some soils contain high numbers of indigenous clover rhizobia the introduction of superior nitrogen fixing strains is still considered an important management practice (McInees et al. 2004). However, the inoculant strains may be susceptible to loss of symbiotic traits such as infectiveness and effectiveness due to environmental factors, and may not be competitive with the indigenous strains already present in the soil (Amarger, 1981; Svening et al, 2001; Slattery et al, 2004).

Diversity of *Rhizobium leguminosarum* bv. *trifolii*

The diversity of native soil populations of *R. leguminosarum* bv. *trifolii* is large and has been well documented in several studies (Demezas et al., 1991; Svenning et al., 2001; McInnes et al., 2004). The characterization of those populations has been performed using different strain typing methods depicting a considerable genetic heterogeneity between strains.

Among the most common methods, serotyping has been used to identify isolates, detect nodule occupancy and track the distribution of antigenically distinct strains (McInnes et al., 2004). However, technical concerns and the involvement of animals in antiserum production can deter some researchers from using serological methods (Beck et al., 1993). Studies carried out with *Rhizobium* and *BradyRhizobium* species have shown that individual serotypes may be comprised of more than one strain (Leung et al., 1994).

Other phenotypic approaches for the study of rhizobial diversity have been done using intrinsic antibiotic resistance (IAR). This methodology uses the natural resistance of strains to given levels of various antibiotics for identification (Beck et al., 1993) and the detection of antibiotic markers is considered more practical than other methods because the methodology is simple, reliable and non-expensive (Mueller et al., 1988; Brockman and Bezdicek, 1989; Abaidoo et al., 2002).

Carbon utilization patterns have also been used to distinguish isolates and strains among the *Rhizobiaceae* family. The ability of *Rhizobium* to metabolize a broad range of sugars, organic acids and aromatic compounds is well documented (Stowers, 1985). The studies in carbon nutrition and metabolism in free-living cells has provided a baseline for comparison among strains. For *R. leguminosarum* bv. *trifolii*, the carbon utilization patterns have been used for phenotypic comparison between plasmid-cured strains (Baldani et al., 1992). The BIOLOG substrate utilization patterns have been used to confirm phenotypic similarities between electrophoretic types given by multilocus enzyme electrophoresis (MLEE) (Leung et al., 1994).

Methods for assessing strain diversity within species include the utilization of molecular techniques. Characterization of the *Rhizobium* genome at the molecular level is the most discriminating method for assesses the variability among strains and isolates of the bacteria (Demezas et al., 1991; Thies et al., 2001). Molecular tools for the identification of bacteria are now available and are used routinely in laboratories.

The electrophoretic screening of large plasmids, although limiting the analysis to the extrachromosomal elements, has a particular importance in the screening of *Rhizobium.* These bacteria have large plasmids (> 50 kb in size) that account for a substantial portion of their genome and contain several genes coding for nodulation and

nitrogen fixation (Long, 1989). These plasmids are often present in variable numbers and sizes; traits that provide the basis for an accurate strain characterization when the extracted plasmids are separated by gel electrophoresis (Corich et al., 2001). However, the use of results from plasmid profile analysis to explain diversity in the population of *R. leguminosarium* bv. *trifolii* must be taken carefully, since plasmids maybe transfer from one cell to another and might not be related to chromosomal variation (Ibekwe et al., 1997). Strains may loose or regain those plasmids, and rearrengments or deletions may occur during laboratory cultivation and in the natural environment (Weaver et al., 1990; Martínez-Romero y Caballero-Mellado, 1996; Zhang et al., 2001)

Intraspecific variation and intragenomic heterogeneity are also limitations for the study of diversity and phylogeny of rhizobia (Young and Haukka, 1996). Sequences of the small subunit ribosomal RNA (SSU rRNA) can vary within a single isolate and the species *R. leguminosarum* has a more dramatic polymorphism (Young and Haukka, 1996).

Several authors have constructed phylogenies based on *nod* gene sequences and have concluded that they are not congruent with those based on SSU rRNA. For example the *nodABC* and *D* genes of *R. trifolli* are closer to *R. viciae* and *S. meliloti.* Therefore, some authors have considered that the study of the genes involved in nitrogen fixation must be worked out separately from that of the bacteria that now carry them (Young and Haukka, 1996).

Other methods that rely on the analysis of features distributed over the whole genome of *Rhizobium* include the pulsed-field gel electrophoresis (PFGE) fingerprinting of large DNA fragments obtained by cutting with restriction endonuclease enzymes (Corich et al., 2001). One important disadvantage of this technique is that standard

protocols involve time-consuming steps such as the DNA preparation, lengthy restriction enzyme digestion and extended electrophoresis times, which for *Rhizobium* can take up to 6 days (Corich et al., 2001).

Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between very closely related rhizobial strains (Oliveira et al., 2000). Although RAPD analysis is rapid, inexpensive and easy to perform, RAPD markers have been reported such as poor reproducibility, heteroduplex formation of homologous sequences, and competition between different DNA fragments for amplification which may result in incorrect genotype interpretations (Hansen et al., 1998).

Restriction fragment length polymorphism (RFLP) analysis has been used to establish genetic relationships and to characterize *Rhizobium* strains at the species and higher levels (Laguerre et al., 1996). RFLP analysis can detect mutations or rearrangements in the genome which alter the distribution of specific restriction endonuclease recognition sites within defined regions of the genome. Recently, RFLP analysis has demonstrated the diversity of Sym (symbiotic) plasmid types within naturally occurring populations of *R. leguminosarum* including bv. *trifolii* (Demezas et al., 1991).

Oligonucleotides derived from enterobacterial repetitive intergenic consensus (ERIC) sequences and extragenic palindromic (REP) elements have been found to be very useful for fingerprinting a variety of Gram-negative and Gram-positive bacteria using the polymerase chain reaction (PCR) (Versalovic et al., 1991; de Bruijn, 1992; Leung et al., 1994, Ibekwe et al., 1995; Svenning et al., 2001).

However, strain diversity within species of rhizobia may be high, both genetic and phenotypic (Young and Haukka, 1996). Therefore it is necessary to define the level of diversity which is appropriate to characterize particular genera and strains.

Numerical cluster analysis of an adequate number of strains and comparison with rhizobial type strains can permit the characterization of large populations. Numerical taxonomy combines the classic data on physiology and morphology in a rigorous and less subjective manner that gives quantitative measures of similarity (Young and Haukka, 1996).

Minimum standards for the description of new genera and species of *Rhizobium* are given by Graham et al. (1991). Proper description and classification of the microorganisms should include numerical analysis of colonial and cultural characteristics and the analysis of representative strains by using measures from molecular studies that enable the identification of the microorganism.

Trifolium nigrescens Viv.

Most clovers (*Trifolium* L.) are remarkable nitrogen-fixers and their establishment improves the quality of pasture in both natural and cultivated grasslands in temperate regions. Among the genus, many species are cultivated not only as forage, but also as green manure, soil erosion controllers and weed controllers through plant competition (Evers, 1983; Woodgate *et al.,* 1999).

Ball clover (*Trifolium nigrescens* Viv.) is an annual, non-stoloniferous clover native to Turkey and distributed throughout the Mediterranean, Asia Minor and Caucasus regions (Pryor and Lowther, 2002; Williams *et al.,* 2001; Williams and Williamson, 2001) that has been introduced as a forage legume in sown pastures of the Southeastern region of United States. Due to its morphological traits, this legume has

the potential to become an important component of many forage-livestock systems in the Central and East Texas regions. Ball clover seeds are planted in early fall in prepared seedbeds or by sod-seeding, and the mature plants are grazed during the winter and spring seasons (Bagley *et al.,* 1988), providing high quality forage during the early spring of the year when low temperatures limit the growth of other forage crops.

Trifolium nigrescens is very similar in appearance to white clover. However, this clover presents a pattern of flowering at every node, generating more inflorescences per plant than white clover. It also produces volatile compounds that act as strong insect attractants (Perkins, 1961) and presents a higher resistance to clover cyst nematode pests than white clover, which has been confirmed in *T. repens* x *T. nigrescens* hybrids (Hussain *et al.,* 1997; Abberton *et al.,* 1999). These morphological traits and the fact that this clover is able to hybridize with white clover makes ball clover a good source of germplasm for white clover improvement (Marshall *et al.,* 2002).

Trifolium. nigrescens grows in clay or sandy loam soils, and tolerates a wider range of soil pH than white, arrowleaf (*T. vesiculosum* Savi.) and crimson (*T. incarnatum* L.) clovers (Evers and Smith, 1998; Buttler and Muir, 2003), with values of pH between 6.5 to 8.0. This clover species is fairly adapted to wet sites although it might show reductions in growth under severe flooding conditions (Smith and Pemberton, 1998).

Ball clover is also a reliable reseeder, producing enough hard seed that does not germinate in the off-season so that does not need to be planted every year when properly managed (Buttler and Muir, 2003). The seed yields for this clover are between 100 to over 300 kg/ha (Hoveland and Evers, l995).

Evaluations for forage production and adaptation carried out by the forage legume breeding program at Overton (Texas) provide information about seasonal

production and yields of common ball clover compared with commercial varieties and breeding lines of rose, arrowleaf and crimson clovers. Ball clover production did not show a significant difference in total yields compared to the commercial and breeding lines during annual tests performed from 1985 to 1989, and was the highest during the 1987-88 growing season (Smith and Gilbert, 1986; Smith *et al.,* 1988; Smith *et al.,* 1989; Smith *et al.,* 1992). For this legume, the nitrogen fixation estimated is 95 kg N/ha $^{-1}yr^{-1}$, which falls in the range of N_2 fixation by other important leguminous crops (Evers and Smith, 1998).

MATERIALS AND METHODS

Sampling of soil

Topsoil samples were collected from a depth of 0 to 15 cm (6 inches depth) from a fine sandy loam of the Zack series (Thermic Udic Paleustalfs) near Iola (Texas) under permanent pasture of ball clover, coastal bermuda, alicia and crabgrass. The ball clover in this location had been growing for over 20 years. By using a spade, three fractions of topsoil were removed from within each of five sample points spaced 20 m apart along a transect running diagonally across a 1 ha. ball clover paddock. A composite soil sample was made from the five subsamples and kept at 4°C until the experiments were initiated. Dr. Gerald Evers (Texas A&M University Agricultural Research and Extension Center at Overton) collected a topsoil sample from a fine sandy loam of the Kirvin series (Thermic Typic Hapludults) near Kilgore (Texas) where ball clover has been growing for 3 years. Eight to ten subsamples were taken randomly from the ball clover paddock and mixed to obtain a composite sample. This sample was kept at 4°C until the experiments were initiated. The gravimetric moisture content of each soil sample was determined by drying the soil at 120°C for 72 hours.

Rhizobium isolation from soil

Common ball clover seeds supplied by the Texas A&M University Agricultural Research and Extension Center at Overton were planted in plastic containers filled with 150 g of soil from ball clover crops. Seeds were scarified by light rubbing with fine sand paper and a surface-sterilization procedure was carried out using 95% ethanol and 1% sodium hypochlorite as described by Weaver and Graham (1994). Due to the low germination percentage of common ball clover, between 10 and 15 seeds were sown at

planting depths between 5 and 10 mm. The plants were grown on a light table at 28°C with a 16 hour photoperiod and watered as needed with a modified Evans' nitrogen-free nutrient solution (Weaver and Graham, 1994).

The emerging nodules were harvested from healthy plants 30 days after sowing. Plant roots were rinsed in tap water to remove loosely adhering soil. Eight to ten healthy nodules were removed from each plant with forceps and surface sterilized first with 95% ethanol and then with 5.25% sodium hypochlorite (Weaver and Graham 1994). Individual nodules were crushed in sterilized water and the suspension streaked onto yeast extact-mannitol (YEM) congo red plates and incubated for 4 days at 28°C. One single colony was taken from each nodule extract directly or after purification through subsequent streaking.

Slants of YEM medium were routinely used for maintenance of the parent cultures of rhizobia (Weaver and Graham, 1994). Bacterial cultures were incubated at 28°C unless otherwise stated. The purity of the bacteria was checked by repeat streaking as well as by microscopic examination with Gram staining.

In order to compare the phenotypic traits of the field isolates with a commercial strain, a pure culture of *R. leguminosarum* bv. *trifolii* was isolated from an inoculant known to be effective in symbiotic nitrogen fixation with white, red, ladino, subterranean and berseem clover (HiStick - Becker Underwood, Inc. I.A. U.S.A.).

All the obtained isolates were tested for nodulation in the host plant before and after the series of experiments. The first authentication test was performed using surface-sterilized ball clover seeds planted onto nitrogen-free agar slopes in 20 x 150 mm test tubes as described by Pryor and Lowther (2002). Five days after planting, seedlings were inoculated with 1 ml of YEM broth containing approximately 10^7 cfu ml^{-1}.

The concentration of the inoculant was adjusted by direct count of four different bacterial suspensions using a Petroff-Hausser Bacteria Counter (C. A. Hausser & Son, Phila., U.S.A.), and then determining the respective absorbance at 540 nm (O.D.= 0.35) with a Turner spectrophotometer model 350 (G. K. Turner Associates, Calif., U.S.A.). After inoculation, tubes were placed in a constant temperature water bath at 25°C on a light table, with 16 hours of light per day. Twenty-five days after inoculation, plants were removed from the tubes and the presence or absence of nodules assessed.

The second authentication test was performed using surface-sterilized ball clover seeds planted into a mixture of sterilized sand-vermiculite (1:2 vol/vol) in 20 x 150 mm test tubes supplemented with 20 ml of modified Evans' nitrogen-free nutrient solution (Weaver and Graham 1994). After germination, the seedlings were inoculated with 1 ml of YEM broth containing approximately 10^7 cells ml^{-1}. Plants were grown under natural light conditions at room temperature (20°C). The presence or absence of nodules was assessed after 28 days.

For both authentication procedures, three replicates per isolate were prepared. Uninoculated plants were used as negative controls to check for cross-contamination. Inoculation with an isolate obtained from a commercial inoculant (HiStick - *Rhizobium leguminosarum* bv. *trifolii* strain, Becker Underwood, IA, U.S.A.) was used as a positive control.

Growth characteristics

Starter cultures of the isolates were grown at 25°C in YEM broth with agitation at 150 rpm for 24 hours, and then diluted ten times in fresh medium. Diluted cultures were aliquoted (150 µl) into single wells of a flat-bottomed 96-well plate (Corning Inc. N.Y., U.S.A.). Growth curves were generated in an ELX808 Microplate Reader (Bio-tek

Instruments, Vt., U.S.A.). Absorbance readings (590 nm) were measured at 1-hour intervals over 48 hours. The temperature inside the instrument chamber was 25°C, and the samples were agitated before each reading. Growth curves were displayed with Excel software from Microsoft, Inc. (Seattle, Wash., U.S.A.). Differences in growth were analyzed by plotting the absorbance data on a logarithmic scale.

Three growth parameters were calculated for each isolate: the maximum growth (*ΔOD*), defined by as the difference between OD maximum and OD minimum after 20 hours of culture (Maurice et al. 2001), the maximum growth rate (μmax) determined by linear regression as the slope of the line when the bacteria grew exponentially (Zwietering et al, 1990) and the mean doubling time (tD). The tD was defined as the time elapsed between the OD value at the beginning of the exponential growth and the double of this OD value, and was calculated by converting the OD values into Log_2 values. By plotting the Log_2 OD values as function of time, the inverse of the slope provided the time required for the culture to increase its density by 1 Log_2 OD. Since it is Log_2, a change of 1-absorbance units means the absorbance has doubled (Singleton, 1999).

Characterization of carbon utilization patterns

BIOLOG GN2 MicroPlate™ system (BIOLOG, Inc., Calif., and U.S.A.) was used to produce metabolic fingerprints by testing the ability of the microorganisms to utilize or oxidize 95 carbon sources. This assay used the reduction of tetrazolium violet to colorimetrically detect the respiration of cells (Bochner *et al.*, 2001).

Cultures of the 43 field isolates and the commercial inoculant strain were grown during 72 hours in YEM agar and uniform suspensions were prepared by suspending the cells in BIOLOG gelling inoculant fluid (BIOLOG, Inc., Calif., U.S.A.). Cell

suspensions for each isolate were standardized to an absorbance value of 0.3 at 595 nm. The suspensions were transferred to a multiwell inoculator plate, and then a 150-µl portion was inoculated into each well of the BIOLOG MicroPlate™. The plates were incubated at 28°C for 72 hours under static conditions, and the respiratory responses were evaluated using an ELX808 Microplate Reader (Bio-tek Instruments, Vt., U.S.A.).

Either a negative respiratory response or a positive respiratory response was recorded for each of the substrates using the MicroLog™ Software (BIOLOG, Inc., Calif., U.S.A.). The data were converted to a two-dimensional binary matrix (1 for positive respiratory response; 0 for no response) for further analysis of the carbon utilization pattern of the isolates.

Intrinsic antibiotic resistance profiles

Resistance to antibiotics was tested on YMA agar supplemented with one of the following antibiotics (µg ml^{-1}): neomycin 12.5, 25: vancomycin 25, 35, 50; kanamycin 10, 25, 40; erythromycin 25, 50; rifampicin 10, 25, 50; streptomycin 12, 25; nalidixic acid 500; tetracycline 1, 5. The test for each antibiotic was carried out in triplicate.

Starter cultures of the isolates and the commercial strain were grown in YEM broth at 25°C and agitation at 150 rpm for 24 hours. Aliquots (150 µl) were transferred onto single wells of a flat-bottomed 96-well plate (Corning Inc. N.Y., U.S.A.). A 96 pin replicator (Nalge Nunc Int. Corp. Ill., U.S.A.) was dipped in 95% ethanol, flame-sterilized, and then dipped into the wells of the 96-well plate containing bacterial suspensions. Forty-five drops (1µl per drop) were transfered from the replicator pins into the YEM reception plate with the corresponding antibiotic concentration.

The plates were incubated at 28ºC during 72 hours and plates containing basal YEM medium were used as controls. Isolates were considered resistant when growth

was similar to the growth in the control plates and sensitive when little or no growth was detected. The data were converted to a two-dimensional binary matrix (1 for normal growth response; 0 for little or any growth response) for further analysis of the intrinsic antibiotic resistant profile of the isolates.

Physiological test for salt, acidity and temperature tolerance

Starter cultures of the isolates and commercial strain were grown in YEM broth at 25℃ and agitated at 150 rpm for 24 hours. Following the same procedure described above, aliquots (150 µl) of each isolate suspensions were transferred onto single wells of a 96-well plate. Using the pin replicator, drops of the cell suspensions were transfered into YEM reception plates with varying pH or salt concentration and performed by triplicate.

Differences in sodium chloride tolerance were tested in YEM agar supplemented with NaCl at a concentration of 0.2%, 0.25%, 0.3%, 0.35% and 0.4% (wt/vol). Differences in acidity tolerance were tested in YEM agar adjusted at pH 3.8, 4.0, 4.3, 4.8 and 5.0 using sulfuric acid (1N).

All the plates were incubated at 28ºC during 72 hours and plates containing basal YEM medium were used as controls.

Differences in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 30°, 35°, 36°, 37°, 38° and 40℃. Control plates were incubated at 28℃.

Isolates were considered salt-tolerant, resistant to low acidity and temperature resistant when growth was similar to the growth in the control plates. The data were converted to a two-dimensional binary matrix (1 for normal growth response; 0 for little or any growth response) for further analysis of the physiological profile of the isolates.

Infection and nodulation of subterranean clover

In order to assess the infectivity of the isolates on subterranean clover, seed of accessions of *T. subterraneum* Denmark N609 provided by Texas A&M University Agricultural Research and Extension Center at Overton were surface-sterilized and transferred to growth pouches containing nitrogen-free nutrient solution according to the methodology described by Weaver and Graham (1994) using three replicates per inoculant treatment with the isolates obtained from ball clover nodules. Seedlings of subterranean clover were inoculated with 1 ml of cultures of the isolates grown at 25°C in YEM broth with agitation at 150 rpm for 48 hours.

Plants were grown under natural light conditions at room temperature (20°C). The presence or absence of nodules was assessed after 4 weeks. Plants inoculated with the commercial isolate were used as positive controls for adequate nodulation conditions and uninoculated plants were used as negative controls to check for cross-contamination.

Numerical analysis

All the collected data were combined in a final matrix containing 110 traits for the 44 isolates and the relationships between isolates with regard to their response were analyzed using the SIMQUAL (Similarity for Qualitative data) subrutine of NTSYS-pc (Exeter Software Co., N.Y., U.S.A). Isolates were grouped by the unweighted paired group mean average (UPGMA) clustering.

RESULTS

Rhizobium isolation from soil

A total of forty-three isolates were obtained from the soil samples of ball clover pastures. After isolation and purification, 26 isolates were obtained from the Iola soil sample and the remaining 17 were obtained from the Kilgore soil sample. For the first authentication test, no differences in plant growth were evident. The uninoculated plants used as controls did not show nodule formation and started to show chlorosis and wilting after the first 2 weeks of the experiment. The second test confirmed that the isolates were able to nodulate the host plant and the uninoculated plants did not form nodules on the roots. However, for this experiment, no visual differences in growth were evident among the inoculated and uninoculated plants.

Growth characteristics

All 43 isolates recovered from nodules of the trap host species showed the same colony type. The coloration was milky-white translucent, with a circular shape, shiny and raised. All the isolates copiously produced extra cellular polysaccharide slime. The values for the three growth parameters are summarized in Table 1. Under the growth conditions, about 72% of the isolates were fast-growing with a doubling time (tD) lower than 4 hours and the remaining 28% were slow-growing ones with a tD greater than 10 hours. These results agree with the observations of growth made in YEM solid media, where the same isolates that showed the slowest tD in the micro-culture were the isolates that showed the smallest colonies after 72 hours of incubation at 25°C.

TABLE 1. Growth parameters of isolates during microculture in YEM broth[a].

Isolate	Maximum Gowth *ΔOD* (OD units)	μmax (OD units)	tD (Hours)
1	0.07	0.004	21.3
2	0.20	0.011	13.4
3	0.15	0.008	15.7
4	0.16	0.009	16.0
5	0.09	0.005	17.8
6	0.15	0.008	14.2
7	0.18	0.010	12.3
8	0.13	0.007	16.1
9	0.36	0.067	2.0
10	0.40	0.075	1.6
11	0.40	0.068	1.8
12	0.37	0.077	1.9
13	0.31	0.066	1.9
14	0.42	0.069	2.1
15	0.43	0.064	2.4
16	0.16	0.009	13.3
17	0.34	0.056	2.7
18	0.36	0.070	1.7
19	0.46	0.088	1.7
20	0.34	0.071	2.0
21	0.41	0.078	1.8
22	0.37	0.069	2.0
23	0.28	0.025	3.7
24	0.10	0.006	16.0
25	0.31	0.060	2.4
26	0.38	0.083	1.6
27	0.38	0.087	1.4
28	0.38	0.079	1.6
29	0.42	0.094	1.4
30	0.40	0.086	1.5
31	0.32	0.046	2.8
32	0.17	0.009	13.1
33	0.28	0.042	3.3
34	0.32	0.063	1.7
35	0.37	0.073	1.7
36	0.38	0.055	2.3
37	0.40	0.069	2.1
38	0.35	0.041	3.0
39	0.12	0.007	15.2
40	0.43	0.077	2.0
41	0.34	0.079	1.7
42	0.44	0.080	1.8
43	0.42	0.082	1.9
44[b]	0.41	0.078	1.9
45[c]	0.30	0.056	2.3

[a] Growth parameters determined in microculture (150 μL) after 20 hours of incubation at 25ºC
[b] Reference commercial strain
[c] *Pseudomonas fluorescens* biotype G

A model of the growth curve (Fig. 1) summarizes the two tendencies of growth according to the calculated μmax for isolates No. 13 (fast grower) and No. (slow grower). The isolates with a μmax lower than 0.03 OD units (slow-growing isolates) showed a constant increment in cell density during 20 hours. The isolates with a μmax greater than 0.08 OD units (fast-growing isolates) showed an exponential growth phase between the second and fifth hour, with a marked increase in cell density. After this period, the slope decreases and therefore, the increase in OD units per time decreases.

The maximum growth expressed as *ΔOD* was correlated to the maximum growth rate (μmax) (R^2 = 0.98) and with the doubling time (R^2 = - 0.95). Fast-growing isolates showed the maximum yield after 20 hours (*ΔOD*), whereas slow-growing ones showed the lowest *ΔOD* and therefore, the lowest yield after 20 hours. According to the growth parameters, the reference strain of *Rhizobium leguminosarum* bv. *trifoli*i and the *Pseudomonas* strain fall into the fast-growing category.

Characterization of carbon utilization patterns

From the 95 carbon sources tested, only 8 carbon sources were assimilated by all isolates (Gentiobiose, a-D-glucose, m-Inositol, Lactulose, D-Mannitol, D-Melibiose, L-Histidine and Glycerol) whereas 20 were not metabolized by any isolate (a-Cyclodextrin, D-Glucoronic Acid, α-Hydroxybutyric Acid, Itaconic Acid, α -Ketobutiric Acid, α -Ketovaleric Acid, Propionic Acid, Sebacic Acid, D-Alanine, Glycyl-L-Aspartic Acid, Glycyl-L-Glutamic Acid, L-Leucine, L-Phenylalanine, D-Serine, L-Threonine, Thymidine, Phenylethylamine, Putrescine, 2,3-Butanediol and D-L- α -Glycerol Phosphate). Of the remaining substrates, α -Ketoglutaric Acid was assimilated exclusively by isolate No. 33. Glycogen was assimilated only by isolate No. 25, and Tween 40 was assimilated only by isolate No. 9 (Table 2).

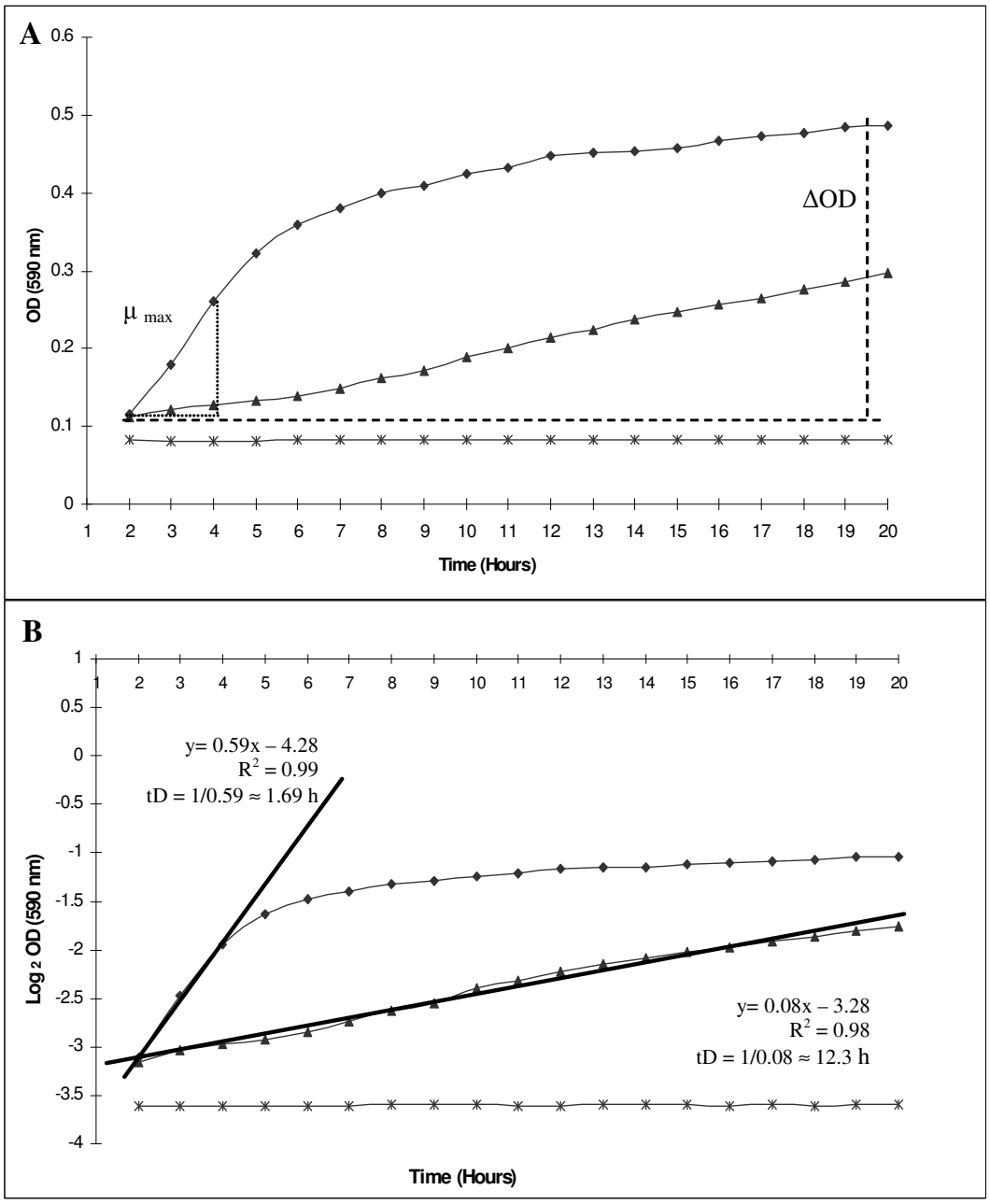

FIG.1. Growth curves representing the two types of growth of the rhizobial isolates recovered from ball clover. Fast-growing rhizobia are represented by isolate No. 35 (◆) and slow growing rhizobia are represented by isolate No. 7 (▲). Uninoculated micro-culture was used as the control (∗). The arithmetic growth curve of absorbance (OD 590 nm) as a function of time (A) allows the calculation of *ΔOD* and μmax. Plotting the Log_2 of absorbance (OD 590 nm) as a function of time (B) allows the calculation of tD. Each point represents the mean of three replicates.

TABLE 2. Carbon utilization patterns of rhizobial isolates recovered from ball clover.

Carbon Source	*T. nigrescens* ISOLATES[a]																																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44[b]	45[c]
Carbohydrates																																													
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Adonitol	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	+	+	-
β-Methyl-D-Glucoside	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-Cellobiose	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Psicose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Raffinose	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
I-Erythritol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-
Lactulose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L-Arabinose	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
L-Fucose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Methyl Pyruvate	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
m-Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Mono-Methyl-Succinate	-	-	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	+	+	-	-	-	+	+	-	-	-	+	+
N-Acetyl-D-Galactosamine	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	-
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Turanose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Xylitol	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	-	+	-	+	+	-	+	+	+	+	-	+	+	+	-
Carboxilic acids																																													
Acetic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
α-Ketoglutaric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Cis-Aconitic Acid	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	+
D,L-Lactic Acid	-	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-	+	+	-	+	+	+	-	-	+	+	+	+
D-Galactonic Acid Lactone	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+
D-Galacturonic Acid	-	-	-	-	-	-	+	-	-	+		+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D-Gluconic Acid	-	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	+
D-Glucoronic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D-Glucosaminic Acid	-	+	-	-	+	-	+	-	+	-	-	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	-	+
D-Saccharic Acid	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Formic Acid	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
γ-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
Malonic Acid	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+
p-Hydroxyphenylacetic Ac.	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	+	+	-	-
Propionic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Quinic Acid	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Succinic Acid	-	-	-	+	+	+	+	-	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
β-Hydroxybutyric Acid	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+

TABLE 2. *Continued*

Carbon Source	*T. nigrescens* ISOLATES[a]																																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44[b]	45[c]
Polymers																																													
Dextrin	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tween 40	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Tween 80	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Amines/amides																																													
2-Aminoethanol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	+
Glucoronamide	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L-Alaninamide	-	+	-	-	-	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-
Putrescine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Succinamic Acid	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Amino acids																																													
D,L-Carnitine	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-	+	-	-	-	-	+	+	-	+
D-Alanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
γ-Aminobutiric Acid	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
Hidroxi-L-Proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
L-Alanine	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+
L-Alanyl-Glycine	-	+	+	-	-	+	+	-	+	-	-	-	+	-	+	+	-	+	+	-	+	-	+	+	-	+	-	+	+	-	-	+	-	+	+	-	+	+	+	-	-	+	-	-	+
L-Asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+	+	+	-	+
L-Aspartic Acid	-	-	-	-	-	+	+	+	-	+		-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+
L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L-Leucine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L-Ornithine	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-
L-Proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Pyroglutamic Acid	+	-	-	+	+	+	-	+	+	-	+	-	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	+	+	-	+	+	+	-	+
L-Serine	-	+	-	+	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	+	-	-	+	+	+	-	-	+	-	-	+	-	-	+	+	-	-	+	+	-	+
Miscellaneous																																													
α-D-Glucose-1-Phosphate	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-
Bromosuccinic Acid	-	-	+	+	+	+	-	+	-	+		+	-	-	-	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+
D-Glucose-6-Phosphate	+	+	-	+	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-
D-L-α-Glycerol Phosphate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Inosine	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Uridine	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	+	-	-	+
Urocanic Acid	-	-	-	-	-	+	-	+	-	-	-	-	+	+	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	-	+	+	+	-	-	-	-	-	+	+	-	+

[a] +,-, strains were positive and negative, respectively.
[b] Reference commercial strain
[c] *Pseudomonas fluorescens* biotype G

According to the classification of carbon substrates proposed by Zak *et al.* (1994), the average number of substrates used by *Rhizobium* isolates is more than 50% of all the carbon sources analyzed (Table 3). The most utilized carbon sources were carbohydrates (~87%) followed by amino acids (~46%) and carboxylic acids (~30%).

The isolates were able to metabolize almost all the carbohydrates from the BIOLOG panel. Only three of the 43 isolates did not show metabolic activity for sucrose, and four isolates were unable to metabolize methyl pyruvate. Ninety percent of the isolates were able to metabolize Cis-Aconitic acid, and more than 80% percent of the isolates were able to utilize quinic and succinic acids.

With regard to amino acid utilization as a carbon source, 39 of the isolates (90%) were able to metabolize L-alanine. Among these, 31 isolates were able to metabolize also L-proline, L-glutamic acid and γ-aminobutiric acid.

The results show that more than 50% of the isolates (23) were able to cleave de dipeptide L-alanyl-glycine and therefore, these isolates showed proteolytic activity.

TABLE 3. Average number and corresponding percentage of substrate categories used by *R. leguminosarum* bv. *trifolii* isolated from *T. nigrescens.*

Substrate category[a]	Substrate utilized	
	Average Metabolized	% Metabolized
Carbohydrates (30)	26.16	87.20
Caboxylic acids (24)	7.23	30.13
Polymers (5)	1.25	25.00
Amines/amides (6)	1.33	22.17
Amino acids (20)	9.32	46.60
Miscellaneous (10)	4.11	41.10
Total (95)	49.40	52.00

[a] Numbers in parentheses indicate the number of carbon sources tested in each category

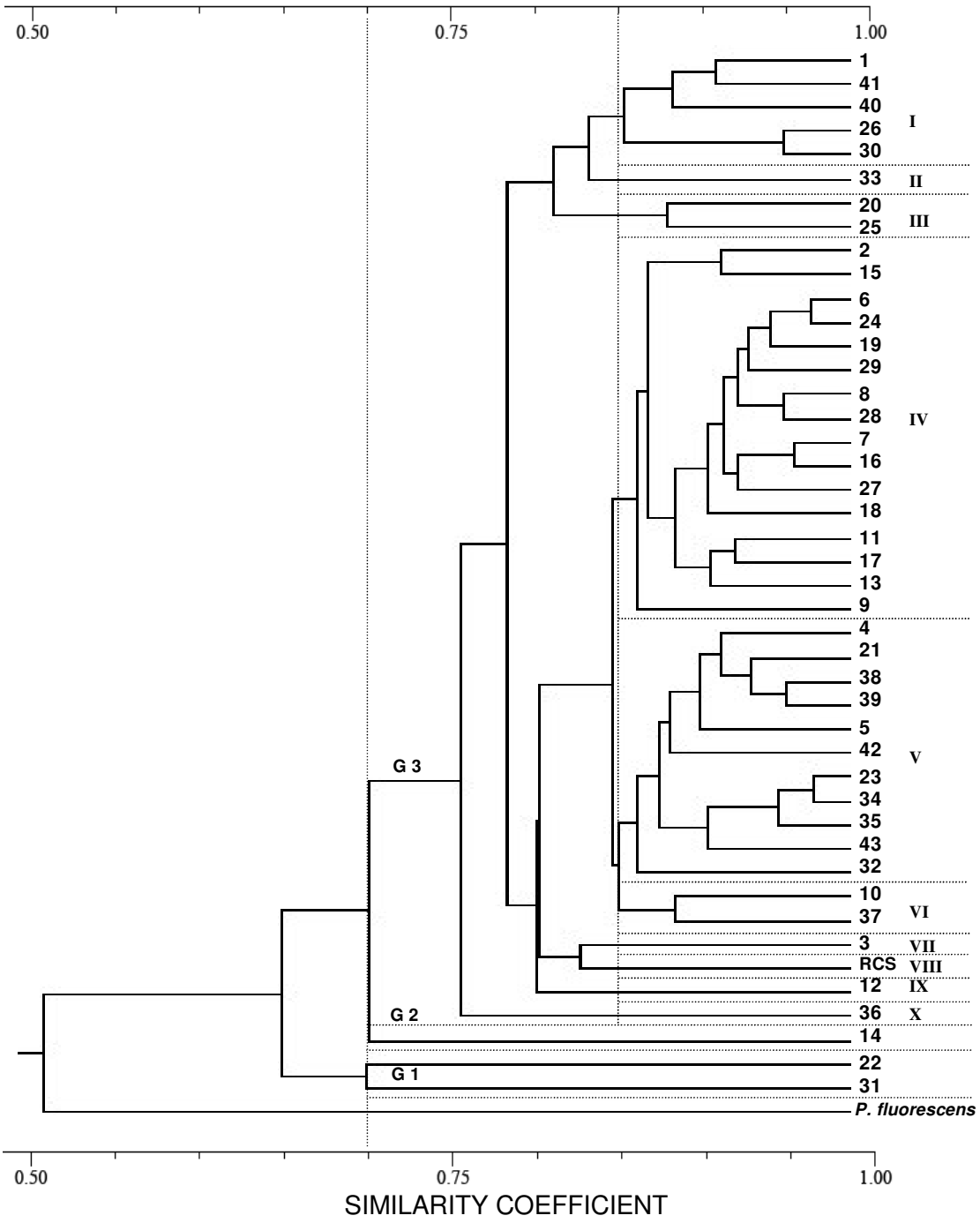

FIG. 2. UPGMA dendrogram generated from BIOLOG analysis of carbon substrate utilization of *R. leguminosarum* bv. *trifolii* isolates from *T. nigrescens* (RCS; Reference commercial strain).

Cluster analysis showed that there were three mayor groups separated at a similarity level of 70% (Fig. 2). Group 1 comprised the isolates that utilized fewer carbon sources when compared to the remaining isolates (No.31 and No. 22). Isolate 31 was unable to utilize any carboxylic acid and metabolized very few amino acids, whereas isolate No. 22 was the only isolate unable to utilize lactose and was able to use only two amino acids as carbon sources. Group 2 contained a single isolate (No 14), unable to metabolize galactose. Group 3 contained the remaining isolates including the commercial strain. This latter group showed a wide diversity and can be classified into 10 clusters at the 85% similarity level due to similarities in the carbon utilization profile.

The strain identified by the BIOLOG system as *Pseudomonas fluorescens* showed a major separation from the remaining isolates (similarity level of 50%).

According to the category of carbon substrate, group 3 was able to utilize more carbohydrates when compared to groups 1 and 2. The clusters IV and V were able to metabolize more carbon sources and utilized a wider variety of substrate categories when compared to the other clusters and groups (Table 4). No separation of isolates by pasture was detected.

TABLE 4. Average number of substrate categories used by the different clusters of *R. leguminosarum* bv. *trifolii* isolated from *T. nigrescens.*

Substrate category[a]	Group 1	Group 2	Group 3 Cluster									
			I	II	III	IV	V	VI	VII	VIII	IX	X
Carbohydrates (30)	19.5	16.0	25.6	25	20.5	26.3	27.2	26	29	27	22	23
Caboxylic acids (24)	4	5	4.2	9	3.5	8.8	8.2	6	4	6	11	4
Polymers (5)	0.5	0	0.2	0	1	0.1	0.3	0	0	0	0	0
Amines/amides (6)	0	0	0.4	0	0	0.8	1	0	0	0	0	0
Amino acids (20)	2.5	10	6.2	7	6	11.2	10.7	9.5	7	6	9	3
Miscellaneous (10)	2	0	3	3	3	2.8	2.7	3	1	1	1	2
Total (95)	28.5	31	39.6	44	34	50	50.1	44.5	41	40	43	32

[a] Numbers in parentheses indicate the number of carbon sources tested in each category

Intrinsic antibiotic resistance profiles

All the isolates showed resistance to low levels of at least one antibiotic (Table 5). As a group, the isolates showed high sensitivity to tetracycline with approximately 90% of the isolates being sensitive to low concentrations and only five isolates were able to grow at the highest concentration of 5 μg ml^{-1}. The isolates also showed low sensitivity to 500 μg ml^{-1} of nalidixic acid, with over 80% of the isolates being resistant to this antibiotic. Fifteen isolates were resistant to high concentrations of two antibiotics simultaneously and isolate No. 40 showed resistance to the highest concentrations of four antibiotics.

When comparing the results of this study with the average Minimum Inhibitory Concentrations (MIC) for natural antibiotics reported for *R. leguminosarum* bv *trifolii* by Hagedorn (1979), very few isolates were able to resist more than the average MIC (Table 6). However, more than 80% of the isolates were able to resist more than ten times the reported MIC of the synthetic antibiotic, nalidixic acid.

A dendrogram developed from similarity between the isolates formed two intrinsic antibiotic resistance (IAR) groups (IAR1 and IAR2) that clustered at the 20% similarity level (Fig. 3). IAR1 was represented by a single isolate (No. 1), which showed resistance to tetracycline, and rifampicin up to a concentration of 25 μg ml^{-1}. At the 85% similarity level, IAR2 separated into 26 clusters. The cluster analysis showed that some isolates have the same antibiotic resistance profile, giving values of 100% of similarity between them.

The antibiotic resistance profiles could not distinguish the rhizobial isolates from the *Pseudomonas fluorescens* strain and no separation of isolates by pasture of origin was detected.

TABLE 5. Differentiating intrinsic antibiotic resistance profiles of the rhizobial isolates recovered from *T. nigrescens.*

Isolate	Antibiotic concentrations (μg ml^{-1})[a]																	
	Neomycin		Vancomycin			Kanamycin			Erythromycin		Rifampicin			Streptomycin		Tetracycline		Nalidixic Acid
	12.5	25	25	35	50	10	25	40	25	50	10	25	50	12	25	1	5	500
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
2	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
3	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	+
4	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	+
5	+	-	+	-	-	+	-	-	-	-	+	-	-	+	-	+	-	+
6	+	-	-	-	-	+	-	-	+	-	+	-	-	+	-	+	-	+
7	+	+	+	-	-	+	+	-	+	-	+	-	-	-	-	+	-	+
8	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-
9	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+	-	+
10	+	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	+	+
11	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+
12	+	-	+	+	-	+	-	-	+	-	+	-	-	-	-	+	-	+
13	+	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	+	-
14	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	+	-	+
15	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	+	-	+
16	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	+
17	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+
18	+	-	+	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+
19	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	+	-	+
20	+	-	+	+	-	+	-	-	-	-	+	+	+	+	-	+	-	+
21	+	-	+	+	-	+	-	-	-	-	+	+	-	+	-	+	-	+
22	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+	+	-	+
23	-	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+
24	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-	-
25	+	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	+
26	+	-	-	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+
27	+	-	-	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+
28	+	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+
29	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	+	-	+
30	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	+	+
31	+	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-
32	+	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	+
33	+	-	+	+	-	+	-	-	-	-	+	+	+	+	-	+	-	+
34	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
35	-	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	+
36	+	-	+	+	-	+	-	-	-	-	+	+	+	-	-	+	+	-
37	+	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	-	+
38	+	-	+	+	-	+	-	-	-	-	+	-	-	+	-	+	-	+
39	+	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+
40	+	-	+	+	-	+	+	+	+	+	+	+	+	-	-	+	-	+
41	+	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-	-
42	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	+	-	+
43	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	+	-	+
44[b]	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	+
45[c]	+	-	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+

[a] +,-, strains were positive and negative, respectively
[b] Reference commercial strain
[c] *Pseudomonas fluorescens* biotype G

TABLE 6. Average Minimum Inhibitory Concentrations (MIC) reported for *R. leguminosarum* bv. *trifolii* and maximum levels of antibiotic resistance for isolates from *T. nigrescens.*

Antibiotic	Average MIC (µg ml-1)[a]	Maximum concentration tested (µg ml-1)	No. of resistant isolates
Aminoglycosides			
Kanamycin	42	40	1
Neomycin	66	25	1
Streptomycin	31	25	8
Tetracyclines			
Tetracycline	8	5	5
Macrolides			
Erythromycin	53	50	1
Miscellaneous			
Vancomycin	17	50	1
Rifampicin	NR[b]	50	8
Synthetics			
Nalidixic Acid	42	500	36

[a] As reported by Hagedorn (1979) for 50 *R. trifolii* isolates from *T. subterraneum*
[b] NR; Not reported

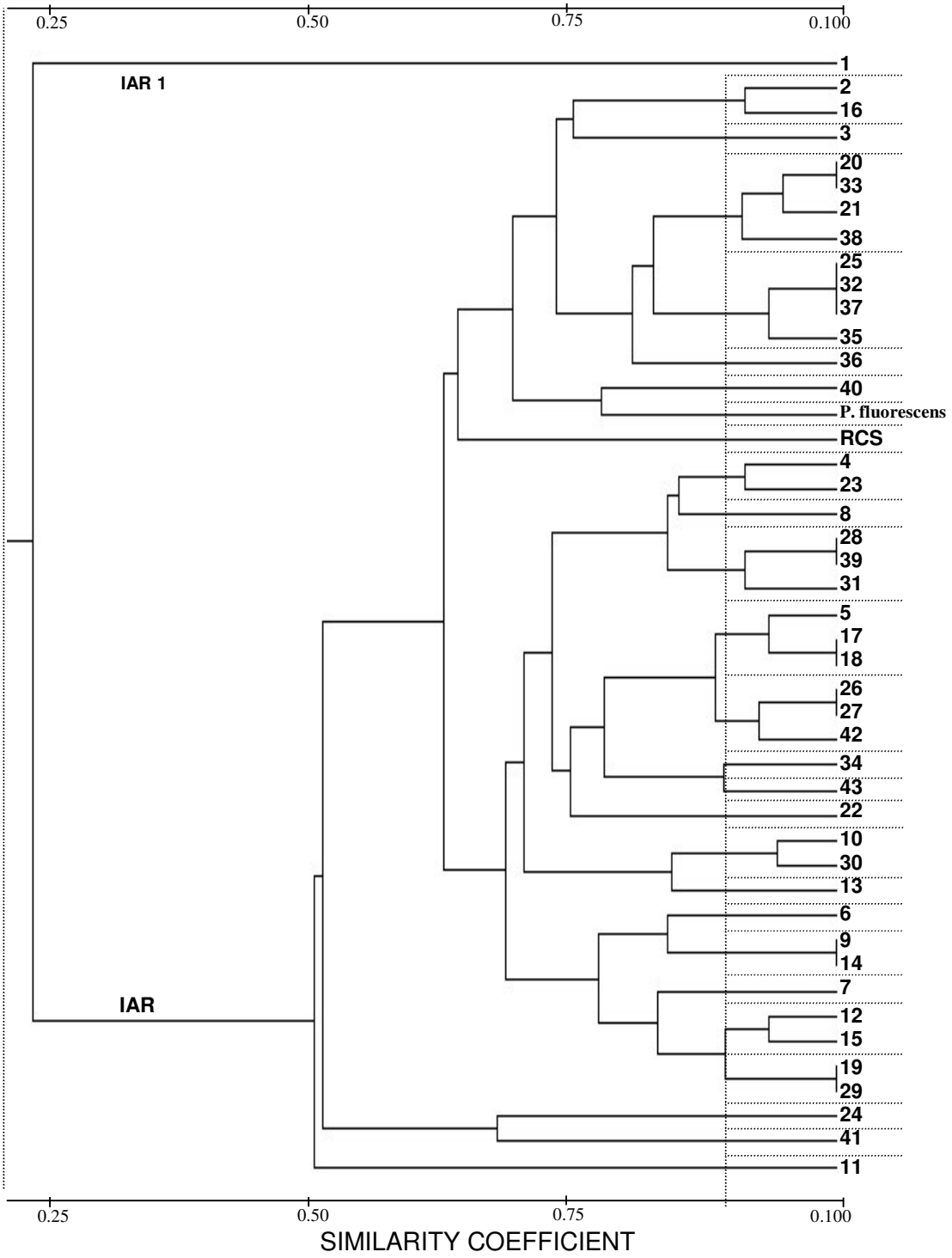

FIG. 3. UPGMA dendrogram generated from analysis of intrinsic antibiotic resistance profiles of *R. leguminosarum* bv. *trifolii* isolates from *T. nigrescens* (RCS; Reference commercial strain).

Test for salt, acidity and temperature tolerance

The physiological traits of the 43 ball clover isolates are summarized in Table 7. All isolates grew in the control cultures (28ºC, pH 7 and 0.01% NaCl). Most of the isolates were able to grow at a NaCl concentration of 34 mM (0.25% w/vol), and only seven were able to tolerate NaCl concentration of 43 mM (0.30% w/vol). The *Pseudomonas* strain was able to tolerate the maximum concentration tested (50 mM).

Forty-two of the isolates were able to grow at pH 4.3 and thirty-seven isolates were able to grow at pH 4.0. Only one isolate (No. 2) was unable to survive below pH 4.5.

The maximum temperature where all the isolates grew was 34ºC and the differentiation according to heat tolerance started at 35ºC. Only one isolate (No. 7) was able to grow at 37ºC.

When analyzed separately, the dendrograms obtained from numerical analysis of each physiological trait did not showed a wide diversity among them. All the physiological traits place the isolates in 4 distinctive groups at 100% similarity level according to their tolerance to NaCl concentrations (Fig. 4A), pH (Fig. 4B) and heat resistance (Fig. 4C). The *Pseudomonas* strain formed a separate group for salt and low pH tolerance. However, according to heat tolerance profile, the *Pseudomonas* strain could not be differentiated from isolate No.7.

Cluster analysis of the three physiological traits showed that there were three mayor groups separated at a similarity index of 75% (Fig. 5). Group 1 comprised the three isolates that were unable to grow above 34ºC and at pH values below 4.3. Group 2 comprised the only isolate unable to grow at pH 4.3, and group 3 was defined by all the isolates that were able to tolerate temperatures from 35ºC up to 37ºC.

TABLE 7. Differentiating physiological traits of the rhizobial isolates recovered from *T. nigrescens*.

Isolate	Physiological trait[a]										
	Temperature tolerance				pH tolerance			NaCl tolerance			
	37°C	36°C	35°C	34°C	4.3	4.0	3.8	0.20%	0.25%	0.30%	0.35%
1	-	+	+	+	+	-	-	+	+	+	-
2	-	+	+	+	-	-	-	+	-	-	-
3	-	+	+	+	+	+	-	+	+	-	-
4	-	-	+	+	+	+	-	+	+	-	-
5	-	-	-	+	+	-	-	+	+	-	-
6	-	+	+	+	+	+	-	+	-	-	-
7	+	+	+	+	+	+	-	+	+	-	-
8	-	+	+	+	+	+	-	+	+	-	-
9	-	+	+	+	+	+	-	+	+	+	-
10	-	+	+	+	+	+	-	+	+	-	-
11	-	+	+	+	+	+	-	+	-	-	-
12	-	+	+	+	+	+	-	+	+	-	-
13	-	+	+	+	+	+	-	+	+	-	-
14	-	-	+	+	+	+	-	+	-	-	-
15	-	+	+	+	+	+	-	+	+	-	-
16	-	+	+	+	+	+	-	+	+	-	-
17	-	+	+	+	+	+	-	+	-	-	-
18	-	+	+	+	+	+	-	+	+	-	-
19	-	+	+	+	+	+	-	+	+	+	-
20	-	+	+	+	+	+	-	+	+	-	-
21	-	+	+	+	+	+	-	+	+	-	-
22	-	-	-	+	+	-	-	+	+	-	-
23	-	+	+	+	+	+	-	+	-	-	-
24	-	+	+	+	+	+	-	+	+	-	-
25	-	+	+	+	+	+	-	+	+	-	-
26	-	+	+	+	+	+	-	+	+	-	-
27	-	+	+	+	+	+	-	+	+	-	-
28	-	+	+	+	+	+	-	+	+	-	-
29	-	+	+	+	+	+	-	+	+	+	-
30	-	+	+	+	+	+	-	+	+	-	-
31	-	+	+	+	+	-	-	+	+	-	-
32	-	+	+	+	+	+	-	+	+	-	-
33	-	+	+	+	+	+	-	+	+	-	-
34	-	+	+	+	+	+	-	+	+	-	-
35	-	+	+	+	+	+	-	+	+	-	-
36	-	+	+	+	+	+	-	+	-	-	-
37	-	+	+	+	+	+	-	+	+	-	-
38	-	+	+	+	+	+	-	+	+	+	-
39	-	+	+	+	+	+	-	+	+	-	-
40	-	+	+	+	+	+	-	+	-	-	-
41	-	+	+	+	+	+	-	+	-	-	-
42	-	+	+	+	+	-	-	+	+	-	-
43	-	-	+	+	+	-	-	+	+	-	-
44[b]	-	-	-	+	+	-	-	+	+	+	-
45[c]	+	+	+	+	+	+	+	+	+	+	+

[a] +,-, strains were positive and negative, respectively
[b] Reference commercial strain
[c] *Pseudomonas fluorescens* biotype G

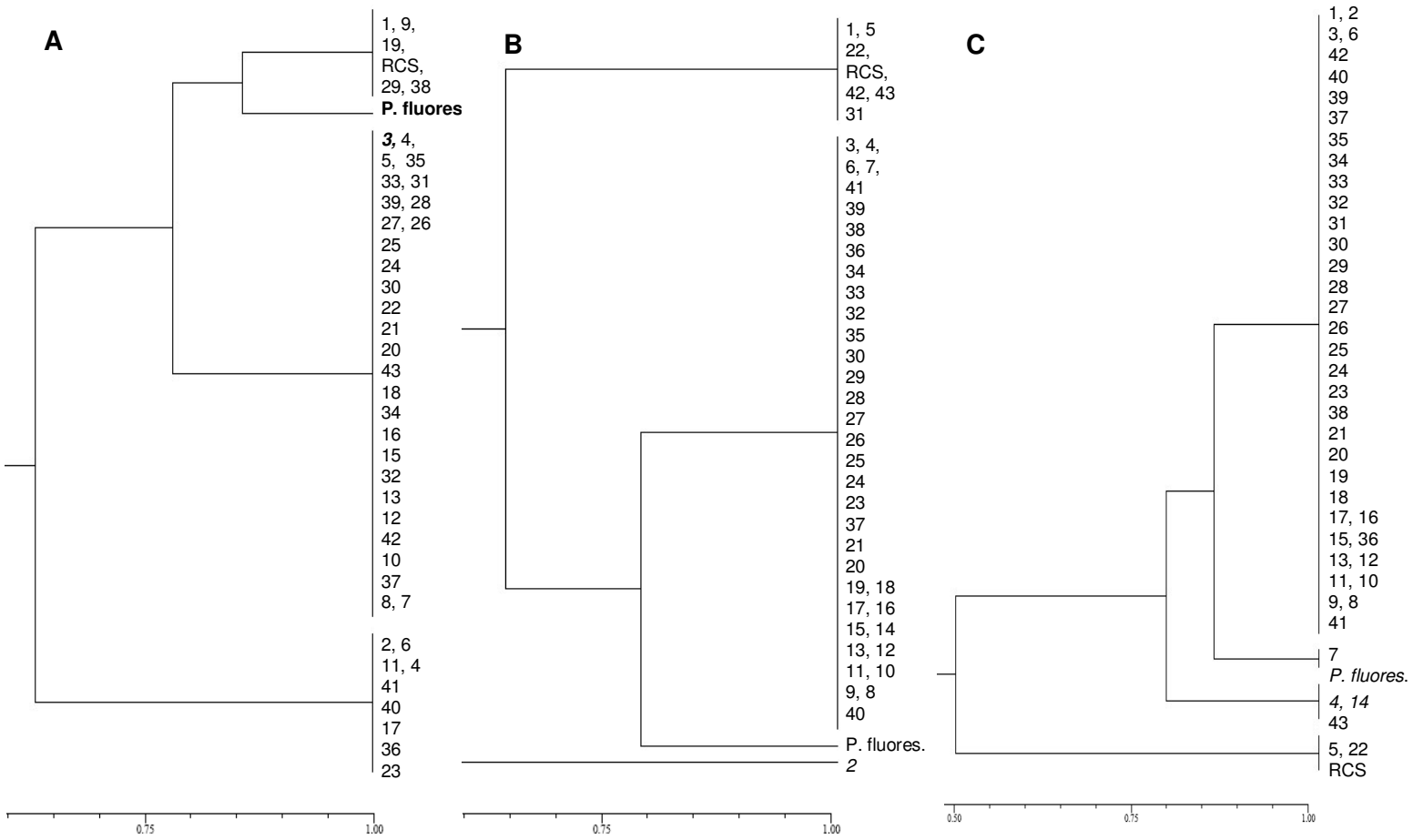

FIG. 4. UPGMA dendrograms of physiological relationships among the *R. leguminosarum* bv. *trifolii* isolates from *T. nigrescens* determined by salt tolerance (A), low pH resistance (B) and heat resistance (C) profiles..

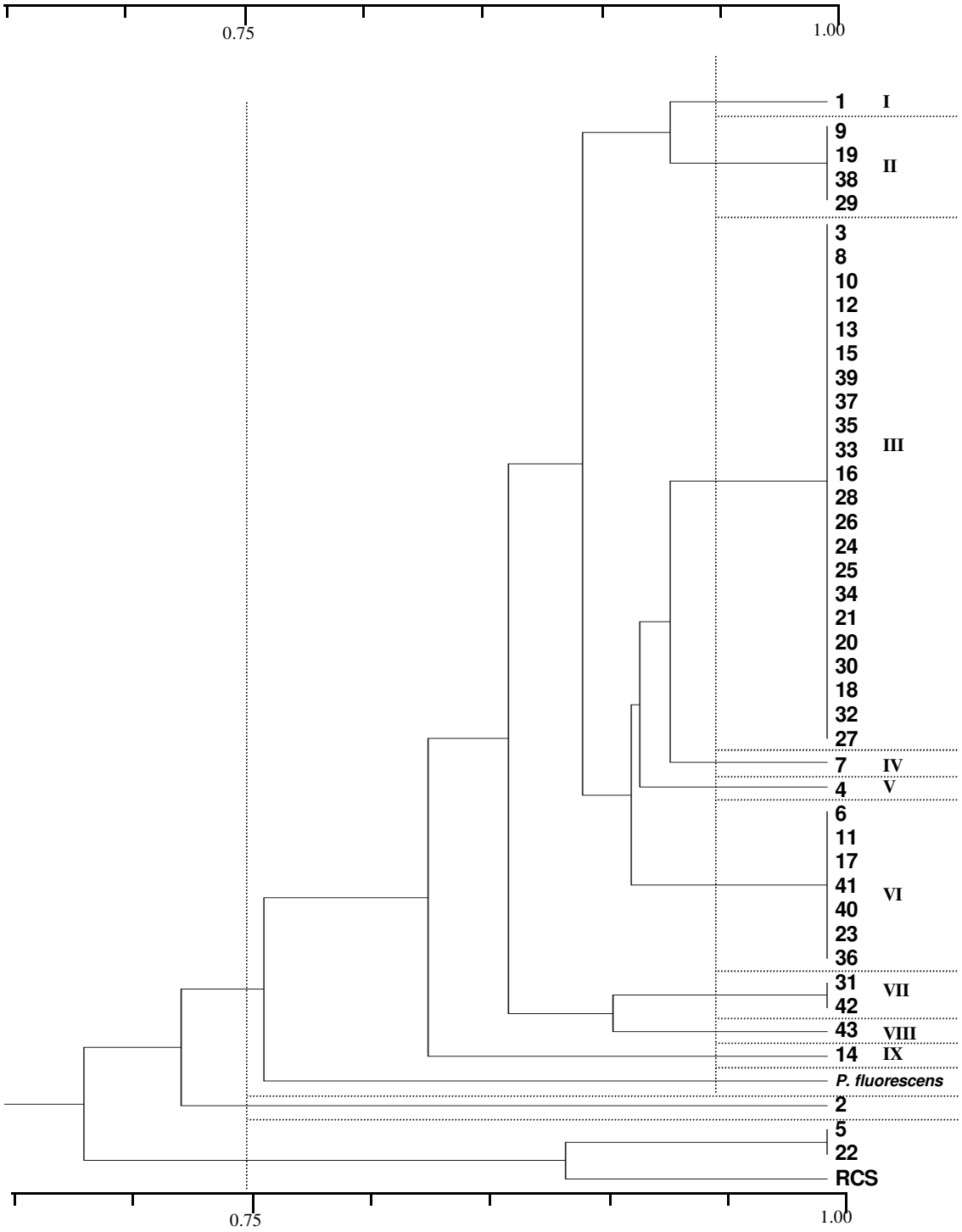

FIG. 5. UPGMA dendrogram generated from analysis of physiological profiles of *R. leguminosarum* bv. *trifolii* isolates from *T. nigrescens* (RCS; Reference commercial strain).

Within this group, 10 clusters were separated at a similarity index of 95%. Six clusters were represented by a unique strain (clusters I, IV, V, VIII, IX and X) and 4 clusters contained between 2 and 29 isolates with the same profile (similarity index 100%). Although the *Pseudomonas fluorescens* strain was the only isolate able to grow at pH 3.8 and tolerate salt concentration up to 60 mM, the cluster analysis of physiological profiles could not distinguish the rhizobial isolates from the *Pseudomonas fluorescens* strain at a similarity index below 75%. No separation of isolates by field of origin was detected.

Numerical analysis

The numerical analysis of 110 phenotypic features allowed the grouping of all isolates into three groups at a similarity coefficient of 75% (Fig. 6). Groups 1 and 2 contained a single isolate (isolates No. 14 and No. 22 respectively), whereas group 3 contained the remaining 42 isolates, including the reference strain.

The separation of isolates No. 22 and No. 14 from the remaining isolates was due to their marked differences in carbon substrate utilization profiles and physiological traits. Isolate No. 22 metabolized fewer carbohydrates and was unable to utilize most of the amino acids when compared to the remaining isolates. This isolate also showed a low tolerance to acidity, salinity, and heat. Isolate No. 14 metabolized fewer carbohydrates when compared to the remaining isolates and exhibited a low tolerance to salinity. The remaining isolates formed a more related group (Group 3). However, the reference strain of *Rhizobium leguminosarium* bv. *trifolii* was the first one to be separated from the other 41 isolates inside Group 3 (cluster 23), because it was the only isolate unable to metabolize D-arabitol, it was one of few isolates able to utilize γ-Hydroxybutyric Acid, and it had poor heat tolerance.

At a similarity coefficient of 90%, the isolates from group 3 formed 23 clusters that contained from one up to nine isolates each, due to their similarities in carbon utilization profiles. Related clusters 3, 4, 5 and 6 (>85 % similarity coefficient) contained the same isolates as cluster V in the BIOLOG dendrogram, showing that these isolates were able to metabolize about 50% of the carbon sources. The same situation occurred with cluster 8, which contained most of the isolates that were in the cluster IV of the BIOLOG dendrogram.

Cluster 1 (Isolate No. 1) was separated from the remaining clusters at the ~76% of similarity level due to the differences in antibiotic resistance profile when compared to the other isolates. Isolate No. 1exhibited high susceptibility to almost all the antibiotics tested except tetracycline.

The *pseudomonas* isolate was separated from the *Rhizobium* isolates at a similarity coefficient of 55%, due to the different carbon utilization profile and high tolerance to salinity, acidity and heat.

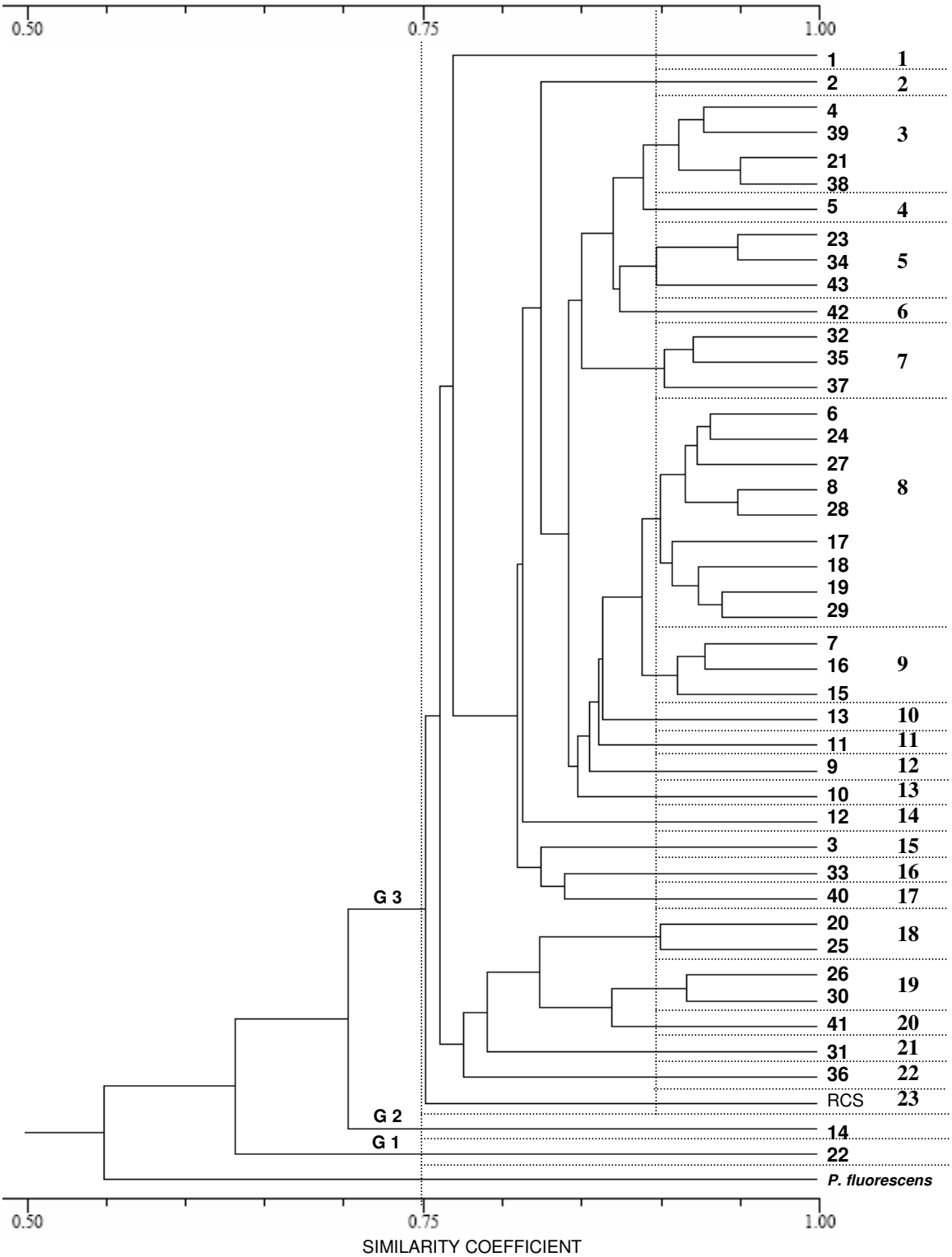

FIG. 6. UPGMA dendrogram generated from analysis of 110 phenotypic traits of *R. leguminosarum* bv. *trifolii* isolates from *T. nigrescens* (RCS; Reference commercial strain).

Infection and nodulation of subterranean clover

The results of cross-inoculation experiments with the isolates obtained from *T. nigrescens* on *T. subterraneum* showed that 42 of the 43 isolates were able to induce nodule formation in subtrerranean clover. Only isolate No. 21 was not able to nodulate subterranean clover plants. However, differences among nodulation responses between subterranean clover and ball clover were evident. Ball clover plants showed deeply red-pigmented nodules whereas the nodules on subterranean clover roots appeared white, which possibly indicated ineffectiveness in nitrogen fixation.

DISCUSSION

The adaptation of *Rhizobium* to the environmental niche of ball clover is critical in the selection for inoculants in order to guarantee their survival in soil. This selection must be performed by testing the ability of the strains to utilize different substrates and to tolerate different environmental conditions. My investigations indicated wide diversity of the 43 isolates with respect to phenotypic characteristics Although different phenotypic approaches provided very different information on the ball clover isolates, they were similarly sensitive in demonstrating the diversity found amongst them.

Growth characteristics

One of the more surprising aspects of my investigation was the difference in generation times of the isolates. Clover rhizobia are considered fast growers (Jordan, 1984), but my results indicate that among the rhizobia that infect ball clover, about 25% of the isolates may be considered as slow growing rhizobia.

The analysis of growth parameters allowed this discrimination between slow and fast-growing isolates. Thirty one of the isolates obtained from ball clover nodules that were classified as fast-growing isolates exhibited doubling times between 1.3 and 3.7 hours, whereas twelve isolates showed doubling times between 12 and 20 hours.

The existence of slow and fast-growing strains among rhizobia isolated from the same host legume has been shown previously for cowpea and soybean *Rhizobium* (Hernandez and Focht, 1984). Slow and fast-growing strains are also present in rhizobial isolates from legumes of the genera *Cicer,* (Maatallah et al, 2002), *Acacia, Sesbania* and *Phaseolus* (Odee et al, 1997).

Other features that allow the identification of rhizobial strains may be related to differences in growth. It has been reported that plasmids can influence the growth of rhizobia in liquid cultures. Moënne-Loccoz and Weaver (1995) found that there are differences in growth among *R. leguminosarium* bv. *trifolii* bearing different types of plasmids. Depending on the type of plasmids and the liquid media, *R. leguminosarium* bv. *trifolii* showed generation times (doubling times) between 1.5 and 6.2 hours (Moënne-Loccoz and Weaver, 1995).

Although the occurrence of slow-growing strains not been described for clover rhizobia, Bernard et al. (1986) reported slow-growing strains of *R. leguminosarium* bv. *trifolii* with doubling times up to 36 hours growing in minimal medium. The slow-growing isolates found in my research were grown in the nutrient medium recommended for *Rhizobium* (YEM broth) under the same conditions as the fast-growing isolates. Therefore, the distinction between fast and slow-growing isolates was not due to the lack of nutrients but to differences in generation rates of the isolates.

Characterization of carbon utilization patterns

With regard to carbon utilization, it has been established that free-living *Rhizobium* is able to utilize a wide variety of carbon sources for growth and energy and have several pathways available for carbon catabolism (Stowers, 1985). All of the isolates obtained from ball clover nodules were able to utilize glucose, mannitol and glycerol as carbon sources. These carbon sources are generally utilized by bacteria of the genus *Rhizobium* (Stowers, 1985). According to Stowers (1985), another carbon source that is utilized by *Rhizobium* is lactose. However, isolate No. 22 was unable to metabolize this carbon source. This feature separated this isolate from the remaining rhizobial strains.

Three of my isolates did not show metabolic activity for sucrose, even though the uptake of this carbon source has been reported to be constitutive for *R. leguminosarium* bv. *trifolii* (Stowers, 1985).

Only ten percent of the isolates obtained in my study were able to use dextrin as a carbon source, which is in agreement with other works indicating that dextrin is rarely utilized by *Rhizobium* (Jordan, 1984).

Pyruvate is a required compound for nitrogen fixation (Mahler and Cordes, 1968). According to my results, four isolates were unable to utilize methyl pyruvate. Another molecule that can substitute for pyruvate in some nitrogen fixation systems is α-ketobutyrate (Mahler and Cordes, 1968). According to my results, none of the isolates were able to utilize the acid form (α-Ketobutiric Acid). However, the plants inoculated with this isolates were able to form nodules and nitrogen deficiency symptoms such as pale leaves were not shown.

The results of the BIOLOG analysis indicated that the cells were unable to utilize pyruvate as the sole carbon source. However, pyruvate can be synthesized in the Enmer-Doudoroff (ED) pathway and by the L-arabinose pathway, in order to be used via tricarboxylic acid (TCA) cycle (Stowers, 1985). Therefore, the inability to grow in methyl pyruvate as the sole carbon source may be related to inability to transport the molecule through the cell membrane.

The transport of C-4-dicarboxilyc acids through the cell membrane of *Rhizobium* has also been reported as critical for nitrogen fixation since they are the carbon and energy source that the plant provides to the bacteria (Finan et al., 1981; Stowers, 1985; Oke and Long, 1999). According to the metabolic profile, 11 isolates were unable to use succinic acid as the sole carbon source. However, the inability of some isolates to

show the colorimetric reaction in the BIOLOG plate with succinic acid as the sole carbon source does not indicate that these isolates were unable to metabolize them, but that they may not be able to transport them across the cell membrane (Stowers, 1985). The relation among succinate (the oxidated form of succinic acid) and nitrogen fixation efficiency has been investigated in detail in *Rhiozobium* bacteroids. Succinate causes division blockage and induces localized deformations that resemble the morphology of the bacteroids inside the clover nodule (Urban and Dazzo, 1982). Bacteroid formation is critical for nitrogen fixation and therefore, the inability of transport and utilization of succinate can be used as a selection criterion for effective nitrogen fixing rhizobia.

Several of my isolates were able to metabolize L-alanine, L- proline and γ-aminobutiric acid, but none of them were able to grow using L-leucine as the sole carbon source. The assimilation and transport of L-alanine, L- proline and γ-aminobutiric by *R. leguminosarum* is carried out by two permeases of the ABC transporter superfamily, the general amino acid permease and the branched-chain amino acid permease, both of which are also able to transport L-Leucine (Hosie et al. 2002). These permeases are required for optimal growth of free-living *R. leguminosarum* on several amino acids as sole carbon and nitrogen sources. However, *R. leguminosarum* has other transporters of alanine (Hosie et al. 2002). The fact that none of the isolates were able to grow using L-leucine as their sole carbon source indicate that for these isolates other transporters may be implicated in the uptake of this amino acid, or the rhizobia that nodulate ball clover might not have the catabolic enzymes required for the metabolism of L-leucine.

My results indicated that more than 50% of the isolates were able to cleave the dipeptide L-alanyl-glycine. Published results suggest that *Rhizobium* is weakly

proteolytic (Jordan, 1984) which explains why my isolates were able to cleave the peptide bond that joins the two amino acids. This is also supported by the fact that all the isolates showed a slow digestion of litmus milk (data not shown), which is a reaction of proteolysis.

The results from the BIOLOG analysis showed that the isolates obtained from nodules of *T. nigrescens* metabolized a broader number of carbon substrates when compared to results of similar studies of carbon utilization patterns carried out by Tesfaye and Holl, (1999) in strains that nodulate other clovers. This ability to metabolize a broad range of carbon substrates may be advantageous for survival in soil.

Previous studies with carbon substrate utilization revealed that clover-nodulating rhizobia are composed of genetically different strains (Baldani et al, 1992; Leung et al, 1994; Testafaye and Holl, 1999). Therefore, the utilization of BIOLOG profiles can be used for differentiation among rhizobial strains that nodulate ball clover.

Intrinsic antibiotic resistance profiles

Resistance patterns of the isolates to eight antibiotics were studied to provide phenotypic data for differentiating the ball clover isolates from each other and to determine the diversity among the isolates. The concentrations used to characterize the isolates were in the range of those used by Hagedorn (1979), who used a similar approach to characterize rhizobial populations isolated from clovers. The generalized sensitivity to Tetracycline in this study agrees with the results reported previously by Jordan (1984) for the genus *Rhizobium* and by Hagedorn (1979) for *R. leguminosarum* bv. *trifolli.*

The markers obtained from the antibiotic resistance profiles can be helpful when selecting microorganisms. More than 60% of the isolates showed resistance to low levels of neomycin (12.5 µg ml-1), and only one of the isolates was able to survive at up to 25 µg ml-1. Neomycin resistant strains had been often related to loss of effectiveness in nitrogen fixation (Amarger, 1981; Jordan, 1984). Therefore, it is important to consider the use of these mutants when dealing with experiments for inoculant selection.

More than 80% of my isolates were resistant to high levels of the DNA gyrase inhibitor nalidixic acid, surpassing about 10 times the average MIC suggested by Hagedorn (1979) for several strains of *R. leguminosarium* bv *trifolii* isolated from *T. subterraneum.* The high resistance to nalidixic acid of the ball clover isolates can be used as a taxonomic feature for differentiation between rhizobia that nodulates ball clover and subterranean clover.

Nalidixic acid has also been used to study the viability of several types of bacteria. Viable *R. leguminosarum* bv. *trifolii* bacteria undergoes cell elongation when exposed to a combination of substrate and nalidixic acid (Bottomley and Maggard, 1990). This feature is useful for direct viable counts of viable-nonculturable bacteria and for determining the viability within a population of soil bacteria. The high resistance of ball clover rhizobia to nalidixic acid can avoid the utilization of indirect viable counts such as plate counts and the most probable number technique for enumeration of viable bacteria in laboratory and field studies

In general, my results support the suggestion of Hagedorn (1979), Kremer and Peterson (1982) and Chanway and Holl (1986) that generalizations regarding the antibiotic resistance patterns for different strains or types of bacteria, specifically *R.*

leguminosarum bv. *trifolii,* are often invalid. The relationships among the isolates provided by the IAR profiles did not overlap with the groups determined by the other methodologies utilized in my investigation. Also, the antibiotic resistance profile was unable to separate the *Pseudomonas* strain from the reference *R. leguminosarum* bv. *trifolii* and the remaining isolates. This indicates that this method is not suitable for determining phylogenetic relationships among the microorganisms, but the diversity in profiles showed that the use of IAR in combination with other methodologies can reflect the diversity of the rhizobia in soil.

Test for salt, acidity and temperature tolerance

Soil temperature, moisture, physical and chemical composition varies within small areas and these variations affect the populations of the soil inhabitants. Therefore, differences in responses towards salinity, acidity and temperature are expected. Physiological traits, in particular those relating to adaptation to pH, salinity and temperature may be distinctive features of the soil bacteria and can depict the diversity of the rhizobia that nodulates ball clover.

Salinity tolerance

According to my results, all of the 43 isolates were able to growth in salt concentrations of 34 mM NaCl (0.20%), equivalent to an electrical conductivity (EC) of 3.3 dS/m approximately. The salt tolerance data reported previously for most clovers is in the range of soils with medium salinity levels (between EC = 1.0 and 2.0 dS/m) (Maas, 1990). My results indicated that ball clover rhizobia tolerated high concentrations of salts.

In legumes, salt stress significantly limits productivity and therefore, the selection of ball clover genotypes that are tolerant to salt stress plays a crucial role for

the establishment of the salt-tolerant and effective strain of rhizobia in saline environments (Zahran, 1999). The utilization of salt-tolerant rhizobial strains as ball clover inoculants may enhance nodulation and nitrogen content under salt stress in reclaimed soils and therefore, further research in selecting salt-tolerant and effective strains of rhizobia is recommended.

It is important to mention that seven of the 43 isolates were able to tolerate NaCl concentrations up to 50 mM NaCl (0.30%), which is equivalent to an EC of 5 dS/m approximately. The ability of some strains of *R. leguminosarium* bv. *trifolii* to growth under NaCl concentrations up to 350 mM in broth culture has been reported previously (Zahran, 1999). However, the legume-*Rhizobium* symbiosis and nodule formation are more sensitive to salt or osmotic stress than are the free-living rhizobia (Delgado et al. 1994; Zahran, 1999; Hussain et al, 2002) and therefore, rhizobial strains that are tolerant to high salinity levels in laboratory may not be effective in nitrogen fixation.

Acidity tolerance

The results showed that several isolates are acid-adapted strains capable of surviving at pH values lower than the pH range between 4.5 and 9.5 reported for the genus *Rhizobium* by Jordan (1984). The fact that different strains of the same species may vary widely in their pH tolerance has been demonstrated previously (Glenn and Dilworth, 1994, Correa and Barneix, 1997).

Some rhizobial isolates can be more sensitive to low pH than their host and this affects the establishment of the symbiosis, limiting the survival and persistence of the rhizobia (Glenn and Dilworth, 1994; Correa and Barneix, 1997; Zahran, 1999).

The performance of some clover-*Rhizobium* symbiosis under acidic conditions is best when the rhizobial strains were isolated from acidic soils (Zahran, 1999).

Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions may help the establishment of the symbiosis and also may improve the acid tolerance of the legume. (Zahran, 1999) In my research, several of the isolates are acid-adapted strains capable of surviving at pH values lower than the pH tolerated by ball clover, and more than 80% of the isolates were able to grow at pH 4.0. Such low tolerance has been previously reported for some strains of rhizobia that nodulates arrowleaf clover, which can survive and even increase in numbers at pH 4.2 (Weaver et al., 1985).

This feature can make these strains more competent in acid soils, thus, It is important to couple the results of my investigation with the selection of breeds or varieties of acid tolerant ball clover, in order to establish and maintain symbiosis in soils with low pH.

The ability of the isolates to utilize a broad range of carbon substrates is also related to the survival of the isolates under acidic environments. Rhizobia are capable of metabolizing different carbon sources so that the products ameliorate the environmental pH (Glenn and Dilworth, 1994). Under acidic conditions the catabolism of organic acids and amino acids leads to alkalinisation (Ibekewe et al, 1997; Glenn and Dilworth, 1994) and this buffering action may help to the establishment of the legume in acidic soils.

According to Jordan (1994), slow growing strains appear to be more tolerant to low pH than fast-growing strains. However, some fast-growing strains are able to grow at a pH as low as 4.0 (Jordan, 1994). Several authors have reported higher tolerance to acidity in slow growing strains due to their ability to alkalinize the culture medium (Glenn and Dilworth, 1994) or due to changes in their cell envelope as a protection

mechanism in stressful environments (Correa and Barneix, 1997). In spite of the fact that no correlation was observed between my slow and fast growing isolates with regard to pH tolerance, five of the eight isolates unable to grow at pH 4.0 were also fast-growing isolates.

Heat tolerance

Almost 90% of my isolates were able to grow at 36°C and only one survived at 37°C. According to Jordan (1984) the maximum temperature reported for some strains *R. leguminosarum* bv. *trifolii* is 38ºC. However, temperature range is highly strain dependent for the genus *Rhizobium* (Jordan, 1984). Other studies with clover rhizobia have demonstrated that certain strains of *R. leguminosarum* bv. *trifolii* are able to grow at various temperatures in artificial cultures, with growth response up to 41°C (Giddens et al. 1982).

Nevertheless, survival under high temperatures does not mean efficiency in nitrogen fixation. Rhizobial strains obtained from hot and dry environments that grew up to 45°C lost their infectiveness and *R. leguminosarum* bv. *phaseoli* incubated at 37°C resulted in ineffective strains (Zahran, 1999). High soil temperatures also delay nodulation (Schomberg and Weaver, 1992) or restrict it to the soil subsurface (Weaver and Graham, 1994).

Although critical temperatures for efficiency in nitrogen fixation for clovers have been reported to be around 30°C (Zahran, 1999), this winter annual clover is exposed to temperatures in the range of 10°C up to 36°C .

Despite the fact that high soil temperatures usually result in the formation of ineffective nodules, several strains of rhizobia have been reported to be heat tolerant and to form effective symbiosis with their host legumes (Zahran, 1999). The selection

of heat resistant isolates may be relevant for cultivation of ball clover, which is sown during late fall when temperatures can reach 35°C.

Infection and nodulation in subterranean clover

In taxonomic studies of nodule bacteria, the host specificity is still used as one of the criteria. However, the cross-inoculation group concept as a major feature for rhizobial identification and classification has been discarded, as the relationship between host range and *Rhizobium* has been shown to be more complex than previously reported (Young and Haukka, 1996; Tesfaye and Holl, 1999).

The results of the cross inoculation experiment showed that the isolates obtained from ball clover were able to infect and nodulate subterranean clover. These results are consistent with the interpretation of Pryor and Lowther (2002) for the inclusion of *T. nigrescens* in the same symbiotic group as *T. subterraneum* since all but one of the isolates was able to form nodules on subterranean clover. However, specificity in the clover-rhizobia symbiosis is not only limited to nodule establishment but also to the effectiveness of nitrogen fixation (Tesfaye and Holl, 1999), and it has been shown that strain competitiveness in soil is independent of effectiveness (Amarger, 1981). Ball clover nodule isolates formed effective symbiosis with the original host and nodulated *T. subterraneum* without efficient nitrogen fixation.

Diversity within rhizobial isolates from ball clover nodules

Clover species rhizobia have been evaluated as a genetically heterogeneous group using a combination of different phenotypic and genotypic approaches (Leung et al., 1994; Hagen and Hamrick, 1996; Tesfaye and Holl, 1999). According to my results, the similarity analysis of 110 phenotypic traits was suitable for determining the genetic diversity among the isolates and provided high definitions of the strains present

in nodules of ball clover. The 43 obtained isolates comprised different phenotypes which can be grouped in 24 clusters that can be treated as 24 different strains that nodulate ball clover. This grouping reduces the number of isolates for testing nitrogen fixation efficiency by half, and taking into account the phenotypic traits associated with inefficiency or resistance to stressful conditions, an adequate selection for studies in inoculant production can be performed.

The comparison between the grouping obtained by the different phenotypic methodologies revealed that the groups and clusters of isolates were all different with different methods, although they were similar in demonstrating the large diversity found amongst the isolates of *R. leguminosarum* bv. *trifolii* that nodulates *T. nigrescens.*

Although the isolates were distributed throughout the clusters and the same isolates did not show a consistent grouping pattern with the reference commercial strain using the different methods, some of the methods could separate the *Pseudomonas* strain as a distinct group different from the rhizobial isolates.

My results agree with the idea that rhizobia are very diverse, both in host range and in physiological properties, and that there is marked heterogeneity within species (Young and Haukka, 1996). This diversity might be explained by the adaptation of rhizobia to changes in soil pH, salinity and temperature, as well as nutrient and carbon sources availability, which are not distributed evenly in soil. Niches may exist were stressful conditions are minimal and the nutrient availability is optimal. These niches may harbor rhizobia that are not resistant to high temperatures, low pH or high salinity. The selection of resistance presumably occurs in locations outside those niches and, as a result of the differential effects of physical and chemical conditions throughout the soil, the diversity of the rhizobia increases.

CONCLUSIONS

In this thesis the genetic diversity of *R. leguminosarum* bv. *trifolii* associated with *T. nigrescens* has been studied using an array of phenotypic differentiating techniques. The results of numerical analysis of carbon source utilization profiles, intrinsic antibiotic resistance profiles, tolerance to acidity, salinity and high temperatures of the 43 isolates obtained from nodules of *T. nigrescens* indicates that there is a wide diversity in bacteria strains able to infect and form nodules in ball clover. The distinctive characteristics of the 43 isolates could warrant identifying them as 24 different strains.

Ball clover rhizobia metabolized a broader range of carbon sources than other clover rhizobia. They also tolerated low pH and survived at high temperatures. These abilities may favor the establishment of the rhizobia and may represent an advantage of these strains over other inoculants used for ball clover.

The analysis of growth characteristics in liquid media showed that the isolates from ball clover nodules can be grouped according two ranges of growth rates: fast-growing (doubling times between 1.4 - 3.7 h) and slow- growing isolates (12.3 - 21.3 h).

Finding many phenotypically diverse rhizobia nodulating *T. nigrescens* indicates that new *Rhizobium leguminosarium* bv. *trifolii* strains may be described after further taxonomic study. The findings of this thesis will contribute to this activity.

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